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DENDRITIC CELL MIGRATION TO DRAINING LYMPH NODE AND T-CELL PRIMING TO BACILLE CALMETTE-GUÉRIN

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Cover picture: Illustrating Dendritic cell migration from skin to draining lymph node after BCG (red) injection in skin.

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Dendritic cell migration to draining lymph node and T-cell priming to Bacille Calmette-Guérin
THESIS FOR DOCTORAL DEGREE (Ph.D.)

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To all Mothers out there!!!

"Talk to yourself at least once in a Day.. Otherwise you may miss a meeting with an EXCELLENT person in this World…"

Swami Vivekananda

ABSTRACT

Dendritic cells (DCs) are unique antigen presenting cells that bridge innate and adaptive immunity. DCs sense invading microbes, sample antigen and migrate to the draining lymph node (DLN) where they prime T cells to the microbe. In spite of many advances in DC biology, little is known about what happens in the DLN after inoculation of *Mycobacterium bovis* Bacille Calmette-Guérin (BCG) in the skin. A BCG footpad infection model was used in this thesis to investigate the above caveat. Some of the important points addressed are the subsets of DCs that migrate from skin to DLN in response to BCG and the factors that regulate this migration. We developed 5- and 6-carboxyfluorescein diacetate succinimidyl ester (CFSE)- based assay to track cell migration from the footpad to the DLN. We found $EpCAM^{low}$ $CD11b^{high}$ DCs to be the main migratory skin DC subset to relocate to the DLN in response to BCG in this model. Migratory DCs were found to home to the T-cell area of the LN, and to co-localize with BCG.

DC and BCG entry into DLN is dependent on IL-1R and MyD88-dependent signaling. The requirement for MyD88 in this process is both DC-intrinsic and -extrinsic. The contribution of the IL-1R ligands IL-1α and IL-1β were found to be redundant for the entry of skin DCs and BCG into the DLN. In addition, DC relocation to DLN is dependent on the BCG inoculation dose, but not on viability of the injected bacilli. Antigen-specific $CD4^+$ T cells expansion to BCG is, however, superior when heat-killed BCG is used. Furthermore, the nematode *Heligmosomoides polygyrus (bakeri),* which establishes a chronic but localized gut infection, was found to reduce BCG-triggered skin DC migration and to mute BCG-specific $CD4^+$ T cell responses in the DLN.

In summary, using a mouse model of BCG infection, this thesis highlights the discovery of a migratory skin DC sub-population that relocates in an MyD88-dependent manner to the DLN in response to BCG and reports on a series of factors that impact on BCG-triggered skin DC migration in this model, including cytokines, BCG viability, BCG dose, and co-infection with a gut nematode.

LIST OF SCIENTIFIC PAPERS

I. **Vishnu Priya Bollampalli,** Lívia Harumi Yamashiro, Xiaogang Feng, Damiën Bierschenk, Yu Gao, Hans Blom, Birgitta Henriques-Normark, Susanne Nylén, Antonio Gigliotti Rothfuchs. BCG Skin Infection Triggers IL-1R-MyD88-Dependent Migration of EpCAMl^{ow} CD11b^{high} Skin Dendritic cells to Draining Lymph Node During $CD4^{+}$ T-Cell Priming.
PLoS Pathogens 2015;11(10):e1005206-

II. Katja Obieglo*, Xiaogang Feng*, **Vishnu Priya Bollampalli***, Isabel Dellacasa-Lindberg, Cajsa Classon, Markus Österblad, Helena Helmby, James P. Hewitson, Rick M. Maizels, Antonio Gigliotti Rothfuchs, Susanne Nylén. Chronic Gastrointestinal Nematode Infection Mutes Immune Responses to Mycobacterial Infection Distal to the Gut**.**
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III. **Vishnu Priya Bollampalli,** Susanne Nylén, Antonio Gigliotti Rothfuchs. A CFSE-Based Assay to Study the Migration of Murine Skin Dendritic cells into Draining Lymph Node During Infection with Bacille Calmette-Guérin.
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IV. **Vishnu Priya Bollampalli**, Jintao Guo, Sara Fernandez Leon, Cajsa Classon and Antonio Gigliotti Rothfuchs. Redundancy in IL-1 and Mycobacterial Viability in BCG-Triggered Dendritic Cell Migration to Draining Lymph Node.
Manuscript.

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Scientific Reports 2017; (7):43989.

CONTENTS

LIST OF ABBREVIATIONS

Ag	Antigen
APC	Antigen presenting cell
BCG	Bacille Calmette-Guérin
BMDC	Bone marrow derived dendritic cell
CARD9	Caspase recruitment domain family member 9
CCR7	CC- chemokine receptor 7
CFSE	5- and 6-carboxyfluorescein diacetate succinimidyl ester
CFU	Colony forming unit
DAMP	Danger associated molecular pattern
DC	Dendritic Cell
DDC	Dermal DC
DLN	Draining lymph node
DTH	Delayed type hypersensitivity
FITC	Fluorescein isothiocyanate
H. polygyrus	*Heligmosomoides polygyrus bakeri*
HES	H. polygyrus excretory–secretory product
HK-BCG	Heat killed BCG
HSV-1	Herpes simplex virus type 1
IFN-γ	Interferon gamma
IL	Interleukin
IVM	Intravital videomicroscopy
LC	Langerhans Cell
LN	Lymph node
LPS	Lipopolysaccharide
MHC	Major histocompatibility complex
mLC	migratory LC
Mtb	*Mycobacterium tuberculosis*
MyD88	Myeloid differentiation primary response 88
PAMP	Pathogen associated molecular pattern
pLN	Popliteal LN

PPD	Purified protein derivative
PRR	Pattern Recognition Receptor
PTx	Pertussis toxin
ROCK	Rho-associated protein kinase
SLO	Secondary lymphoid organs
SWAg	Soluble worm Ag
TB	Tuberculosis
TGF-β	Transforming growth factor β
Th	T helper cell
TLR	Toll like receptor
TNF-α	Tumor necrosis factor alpha
WHO	World Health Organization

1. INTRODUCTION

The immune system mediates resistance to infection and tolerance to self. It can be broadly divided into an innate and an adaptive arm. Cells of innate immunity include phagocytes such as macrophages, dendritic cells (DCs) and neutrophils, and innate lymphoid cells such as natural killer cells. Innate immune cells use germline-encoded receptors to recognize invading microbes. T cells and B cells belong to the adaptive immune system. They have somatically rearranged receptors with randomly-generated specificities. DCs are innate immune cell that have a key role in orchestrating the adaptive immune responses (1). In tissue DC are exposed to antigen which may allow them to undergo maturation and consequently migrate to draining lymph node (DLN), where they can activate or "prime" naïve T cells. Thus, DCs play a central role in initiating adaptive immune responses needed to effectively control microbial growth and to create memory to re-infection. Highlighted below are characteristics of DCs relevance to their migratory and T cell priming capacity, which both are highly relevant to understand the initiation of immune responses to mycobacteria

1.1. DCs

DCs are a heterogeneous population of phagocytes that reside in tissue as well as in secondary lymphoid organs. They are potent antigen presenting cells (APCs), which are involved in promoting both adaptive immune responses to pathogens and in maintaining tolerance to self-antigens (2). Since their discovery by Ralph Steinman in 1973 (3), extensive research has been done on the biology of DCs, characterizing among other things their contribution to immunity, tolerance, vaccination and immunotherapy. These advances have lead to the identification of several distinct DC subsets by the presence of unique surface and intracellular phenotypic markers based on immunological function or anatomic distribution (4).

1.2. Skin and lymph node DC network

DCs express MHC-II and the CD11c integrin (5). Co-expression of MHC-II and CD11c together with additional markers are used to classify DCs further into distinct subsets. Classification of DCs into distinct subsets with different abilities to process and present antigen can be based on their anatomical location, ability to migrate to DLN and commitment

to initiate distinct T-cell effector responses (6). The study of non-lymphoid tissue DCs involves the surfaces that are in contact with the environment, which includes skin and mucosal surfaces. The skin has received a lot of focus since it houses a large population of DCs and is a highly accessible surface for experimentation in laboratory animals. Skin acts as a barrier for physical stress, environmental antigen/pathogens, and chemical agents. Hence, immune cells in skin have to be prepared to distinguish between self and non-self, to induce protective immunity or tolerance (7). DCs in the skin serve as sentinels. They play a crucial role in guarding the host against pathogens from the environment. Langerhans cells (LCs) are typical DC residents of skin. LCs resides in the epidermis and can move to the DLN. The chemokine receptor, CCR7 is needed for the migration of LCs from the epidermis to DLN (8). LCs are identified by surface expression of Langerin (CD207), CD11b, EpCAM (CD326) and Sirpα (7). LCs were previously believed to be the principal skin DC that sense, phagocytize and transport microbial antigen to DLN for the initiation of immune responses (9). However, studies from epidermal infection with Herpes simplex virus type 1 (HSV-1) suggest that LCs are not an important APC for priming T- cell responses (10, 11). Studies show instead that LCs are precommitted to immune tolerance induction (12). Whether LCs are tolerogenic or immunogenic may depend on type of antigens, cytokine profiles in the environment and the maturation status of the cell itself (2).

Dermal DCs (DDCs) are another population of DCs in the skin. They are found in the dermis (as suggested by their name) and like LCs, migrate to DLN via afferent lymphatics (13). DDC sub-populations can be classified on the basis of three surface markers, namely CD103, CD207 and CD11b. Four subsets of DDCs can be identified with these markers: $CD103^+$ $CD207^+$, $CD103^-$ $CD207^+$, $CD207^-$ $CD11b^+$, and $CD207^-$ CD11b DDCs. These markers also identify LCs in the DLN, which are migratory $CD11b^{low\ to\ high}$ and $CD207^{inter\ to\ high}$ (mLCs) (14, 15). DDC migration to cutaneous lymph nodes (LNs) is CCR7-dependent (15, 16). In the LN, DDCs efficiently present antigens (Ags) to $CD4^+$ T cells, which lead to the activation and expansion of the T cells (17-19). During HSV-1 infection, $CD103^+$ DDCs play a unique role in presenting viral Ags to naïve $CD8^+$ T cells (17). $CD103^-$ DDCs also migrate to DLN in response to chemical stress or mechanical injury (20).

LN-resident DCs are classified into two main subsets based on surface expression of CD8 and CD11b; namely $CD8^+$ $CD11b^-$ and $CD8^-$ $CD11b^+$ DCs (5). LN-resident DCs are positioned close to LN conduits in the paracortex of the LN, facilitating them to present soluble antigen from lymph to T cells (21). Also, studies suggest that LN-resident DCs

control the entry of lymphocytes by modulating the maturation state of high endothelial venules (22). $CD8^+$ DCs can cross-present antigens acquired from skin DCs that have migrated to DLN (10). $CD8^+$ DCs and $CD103^+$ DDCs share in common the transcription factors Batf3 and IRF8, which are important in controlling their development (23, 24).

1.3. DC maturation

DCs play an important function in patrolling body surfaces, from where they sample Ags and then traffic to DLN to initiate adaptive immune responses (4). Mechanisms by which DCs sample Ags include phagocytosis (25), endocytosis and macropinocytosis (26). Immature DCs are highly phagocytic compared to its mature DCs (25). Immature DCs are specialized at directly recognizing pathogen- or danger-associated molecular patterns (PAMPs or DAMPs) by their germline-encoded pattern recognition receptors (PRRs). The initial sensing of infection is mediated by such PRRs, which include Toll-like receptors (TLRs), C-type lectin receptors, retinoic acid-inducible gene I-like receptor (RIG-I-like receptors) and nucleotide-binding oligomerization domain-like receptors (NOD-like receptors) (27). Among the above-mentioned PRRs, TLRs are the best characterized. In mouse there are 11 TLR members that are positioned either on the cell surface (TLRs 1,2,4,5 and 6) or on endosomal membranes (TLRs 3,7,8 and 9) (28, 29). TLRs work as homo- or heterodimers, to recognize very divergent PAMPs including components from bacteria, fungi, viruses and protozoans. TLR signaling leads to the recruitment of different intracellular adaptor molecules like myeloid differentiation primary response 88 (MyD88), Toll-interleukin 1 receptor domain containing adaptor protein (TIRAP), Toll-interleukin 1 receptor domain containing adaptor protein interferon-β (TRIF) and TRIF-related adaptor molecule (TRAM) (27, 30). MyD88 is used by all the 11 identified TLRs except for TLR3. MyD88 is important for DC activation and prominent in initiating Th1 response to *Mycobacterium* (31, 32). TLR activation initiates innate immune responses and also regulates adaptive immune responses to pathogens by activation of NF-κB and distinct IRF3 pathways that upregulate pro-inflammatory cytokines and type I IFNs (27). IL-1R signaling also uses MyD88 for switching on the upregulation of inflammatory cytokines (33), which can amplify the adaptive response by attracting and activating innate immune cells like monocytes and neutrophils to the target tissue (34).

Activation of DCs is associated with varied expression levels of integrins, MHC-II and co-stimulatory molecules like CD80, CD86, and CD40 (35). Antigen processing machineries vary between different DC subsets. $CD8^+$ DCs are efficient in processing antigen for presentation on MHC-I, whereas $CD8^-$ DCs excel at presenting antigen on MHC-II (36, 37). Depending on the type of activation stimulus, the functionality of the DC can be either inflammatory or anti-inflammatory.

Interestingly, PRRs expressed on DCs sense several PAMPs from mycobacteria, such as the cell wall components lipomannan (LM), lipoarabinomannan (LAM) and its mannosylated form (ManLAM) and ESAT-6 (secreted protein) (38). The PRRs that are involved in the above recognition include TLR-2 (39), TLR-4 (40), Mannose receptor, DC-**s**pecific intercellular adhesion molecule-3-grabbing non-integrin (DC-SIGN) (41), Dectin-1 (42, 43) and Dectin-2 (44). (Another C-type lectin is Mincle. NOD-2 also recognizes mycobacteria). Mycobacterial PAMPs that make their way into the cytosol, can activate intracellular PRRs, like TLR-9. TLR9 binds to CpG motifs, non-methylated DNA sequences (31). Triggering of PRRs leads to the activation of NF-κB and culminates with the transcription of several genes important for inflammation (30). All these sequential events trigger DC maturation that can be detected by i) upregulation of co-stimulatory molecules, ii) increase in antigen presentation machinery and iii) pro-inflammatory cytokine production like interleukin-1β (IL-1β), interleukin-12 (IL-12) and tumor necrosis factor alpha (TNF-α) (45). Further, IL-12 and TNF-α secretion will induce IFN-γ production from natural killer and T cells (46). IFN-γ also stimulates DCs to increase their phagocytic activity. An activated DC that has migrated to the LN is now capable of activating antigen-specific T cells that can help control infection (47).

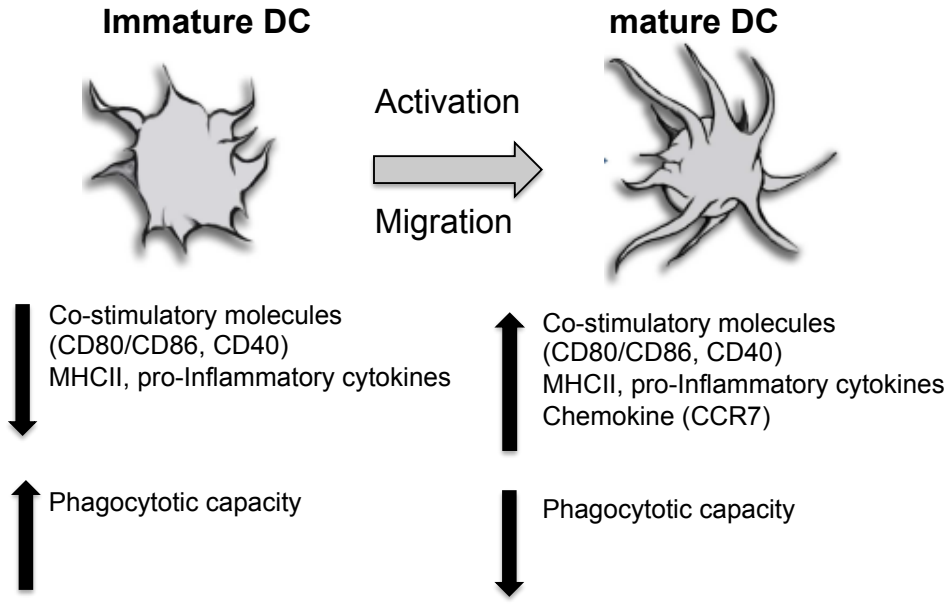

Figure 1. Phenotypic changes that accompany DC maturation. Modified from (48, 49).

1.4. DC trafficking molecules

Trafficking of mature DCs will upregulate chemoattractant-dependent receptors and adhesion molecules that can respond to a broad variety of ligands (50, 51). The migration of DCs to both lymphoid and non-lymphoid tissue depends on the relative expression of chemokine receptors on DCs and the availability of chemokine ligands in the tissue.

1.4.1. Migration of DCs to non-lymphoid tissue

Immature DCs migrate to non-lymphoid tissue by utilizing a variety of chemokine receptor pathways, like CCR2-CCL2 (52, 53), CCR6-CCL20 (53) and CCR5-CCL5 (54, 55). Activated endothelium express E- and P-selectin, which helps in tethering and rolling of immature DCs, monocytes and plasmacytoid DCs (56-58). Irradiation studies confirm that circulating LC migrates to inflamed skin with the help of CCR2/6. Inflammation also boosts expression of the CCR2/6 and corresponding ligands CCL2/7 and CCL20, favoring more LC migration to the inflamed skin (53, 59).

1.4.2. Migration of DC to DLNs

In steady state, DCs in tissue continuously relocate via lymphatic vessels to DLN (60). This trafficking of DCs from tissue is increased during inflammation and infection. The

mobilization of DCs to DLN is a complicated process that involves many steps. Each step is discussed below in the context of skin DC migration.

1.4.3. Mobilization from tissue

The skin microenvironment provides signals for the retention of DCs in the skin (61, 62). It is well established that LCs in the epidermis are anchored with keratinocytes through E-cadherin (63, 64). Selective disruption of E-cadherin junction interactions between LCs and keratinocytes promotes enhanced LC migration by upregulating CCR7 (65). Similar observations are seen when mice that are treated with IL-1β, TNF-α or LPS (66). On the other hand, TGF-β treatment upregulates E-cadherin expression on DC precursors and inhibits their maturation (67) and CCR7 expression (68). After detachment from skin, DCs migrate through the extracellular matrix. DC upregulation of metalloproteinases (MMPs) facilitates their migration through this matrix of collagen, fibronectin and laminin. In particular skin DCs upregulate MMP-2 and MMP-9 (69).

1.4.4. Traversing the afferent lymphatic endothelium

After making their way through the extracellular matrix, skin DCs enter the initial dermal lymphatic vessels by traversing through oak leaf-shaped lymphatic endothelial cells (70). This step requires upregulation of E-selectin and chemokine ligands. Lymphatic endothelial cells stimulated with inflammatory cytokines (TNF-α) upregulate E-selectin, chemokines CCL5, CCL20, and CXCL5, and adhesion molecules ICAM-1 and VCAM-1 (71). Blocking the ability of lymphatic endothelial cells to express adhesion molecules either by neutralizing antibodies or by genetic disruption leads to reduced DDC transmigration of lymphatics (71) and impaired LC migration to DLN (72).

1.4.5. DC motility within SLOs

DCs exit lymphatic vessels and enter the sub-capsular sinus floor of the LN. This is an active process involving CCR7 expression on DC and CCL21 ligand expression on the sub capsular sinus wall of the LN (73). Within the LN, DCs also use CCR7 for migration. DCs depend on CCL21 gradient and are believed to guide them towards the high endothelium venules (HEVs) (74), where expression of CCR7 ligands CCL19 and CCL21 are high.

DC motility behavior relies on amoeboid movement (75). DC amoeboid movement is characterized by actin polymerization events in the leading-end of the cell. This is mediated by Rac1, Rac2 (76), the Rho GTPase family member cdc42 (77) and leads to the formation of protrusions. In rear-end of the DC, actinomyosin contractions are mediated by Rho-associated protein kinase (ROCK). In line, disruption of Rac-1 and Rac-2 leads to ablation of DC migration from skin to LN (76) and inhibition of ROCK significantly reduces the velocity of DCs in the interstitium of the ear skin (78).

To date, CCR7 and its ligands CCL19 and CCL21 is the most well-established chemokine pathway for DC migration from skin to DLN. In support of this statement, DC migration from tissue to LN is reduced in plt/plt mice, which lack CCL19 and CCl21-ser (74, 79, 80). Plt/plt mice have small LNs and a reduced number of migratory skin DCs in skin DLN. Injection of TNF-α in the skin triggers DC migration to DLN (67) and is associated with increased expression of CCL21 by lymphatic endothelial cells. Finally, $CCR7^{-/-}$ mice confirm the key role of this receptor on DCs for their migration from skin to DLN (16, 81, 82). Studies with $CCR7^{-/-}$ and plt/plt mice show the importance of CCR7 and its ligands in the migration of skin DCs to DLN.

However, CCR7 is not the only chemokine receptor that regulates DC migration (83). Adoptive transfer experiments showed although there is uniformly high expression of CCR7 on $CD8^{+}$ DCs, their migration to DLN is reduced when compared with $CD8\alpha^{-/lo}$ DCs which express less CCR7 (84). The above finding suggests that other guiding molecules may be involved in DC migration, such as lipid mediators (85). Indeed, FITC-painting experiments suggest that CXCR4 contributes to both LC and DDC migration to LN in a CCR7-independent manner (4).

1.5. Methods to track the migration:

The development of DC-tracking assays such as FITC-skin painting, discussed below, has helped to uncover the voyage that DCs make from tissue to DLN. Techniques to study DC migration include confocal microscopy, which allows the study of DCs and their location in the tissue and intravital microscopy that allows the study of DC migration in real-time (58, 86). Microscopy can be combined with flow cytometry, which allow thorough characterization of the migrating DC with different surface markers.

DC migration from tissue to LN has been readily studied by applying fluorochromes to the skin surface as a way to label local DCs and then following these labeled DCs by different techniques, such as the ones mentioned above. Injecting fluorescent beads in the skin is one approach, but by far the most commonly used technique is to "paint" the skin with fluorescein isothiocyanate (FITC) mixed with a skin irritant. FITC-labeled LCs and DDCs or monocyte-derived DCs can then be quantified in LN by flow cytometry or microscopy (87, 88) In other studies Carboxyfluorescein succinimidyl ester (CFSE) is given intranasally to label airway DCs (89, 90). Also, antigens or proteins can be flurochrome-coupled and used to study fluorescent populations that become associated with the antigen (91, 92). A potential drawback of the above is to assure the primary location of cell labeling in the tissue. The antigen/flourochorme label may leak due to cell death and other populations may take up the fluorochrome-labeled antigen and also become labeled (89, 92). Another caveat is that the labeling intensity may decrease with time, due to cell metabolism or turnover of the labeled proteins in the cells. Finally, reporter mice where DCs are permanently or conditionally labeled with fluorescent proteins, such as Kaede transgenic mice (93), have become a popular way to track DCs migration *in vivo* (14, 94). A drawback of this approach is or may be that the expression of reporter protein may change as DCs mature.

1.6. Initiation of T-cell response by DCs

DCs in SLOs activate or "prime" naïve T-cells into action. This process requires a combination of three unique signals provided to the T cell by the DC. These signals are commonly referred to as Signal 1, 2 and 3, and trigger T-cell activation, proliferation and differentiation. This is discussed below in the context of $CD4^+$ T cells and summarized as part of Figure 2. Signal 1, or antigen presentation, is the presentation of peptide antigen anchored onto MHC-II. It leads to triggering of the T-cell receptor (TCR) on the T cell. Signal 2 or co-stimulation, is mediated by expression of co-stimulatory molecules CD80, CD86 on the DC and their interaction with CD28 on the T-cell. In the absence of Signal 2, $CD4^+$ T cells become anergic or die (95, 96). Delivery of Signals 1 and 2 enables T-cell expansion and is accompanied by effector cytokine release by the T-cells. Signal 3, or differentiation, is mediated by DC soluble or membrane-bound factors, mainly cytokines, which help commit and "polarize" the expanding $CD4^+$ T cells into a particular T helper (Th) cell lineage. Studies also show that the duration of antigen presentation and the strength of the TCR signal might affect T-cell differentiation (97). The activation of naïve T cells is characterized by sequential events on the T-cells that include the upregulation of CD69,

CD25 and CD44 (98), the downregulation of CD62L (99) and finally, by the production of effector cytokines.

Each Th cell lineage produces a distinct set of effector cytokines that are important in turn for host resistance against different types of microbes. Each lineage is also characterized by the expression of lineage-specific transcription factors (100). These aspects are also summarized in Figure 2 and discussed briefly. IL-12 production favors the differentiation of Th1 cells during priming (101). Th1 cells produce TNF-α and IFN-γ, and through these cytokines, especially IFN-γ, activate the anti-microbial response of macrophages (102). However, there are also reports of IL-12-independent Th1 differentiation and IFN-γ release (103). IL-4 and IL-13 on the other hand, promote the differentiation of Th2 cells. Th2 cells produce IL-4, IL-5 and IL-10 and mediate host resistance to helminths (102, 104) (102, 105, 106). The combination of IL-23, IL-6 and TGF-β can promote the differentiation of Th17 cells. This Th lineage produces IL-17 and IL-22, important in triggering neutrophil responses and host resistance to fungi. TGF-β is also involved in the differentiation of regulatory T cells. These T cells produce IL-10 and TGF-β, and are important in controlling inflammation and dampening immune responses (107, 108). DCs are certainly a major source of IL-12 and IL-23 but interestingly, not for IL-4 and IL-13.

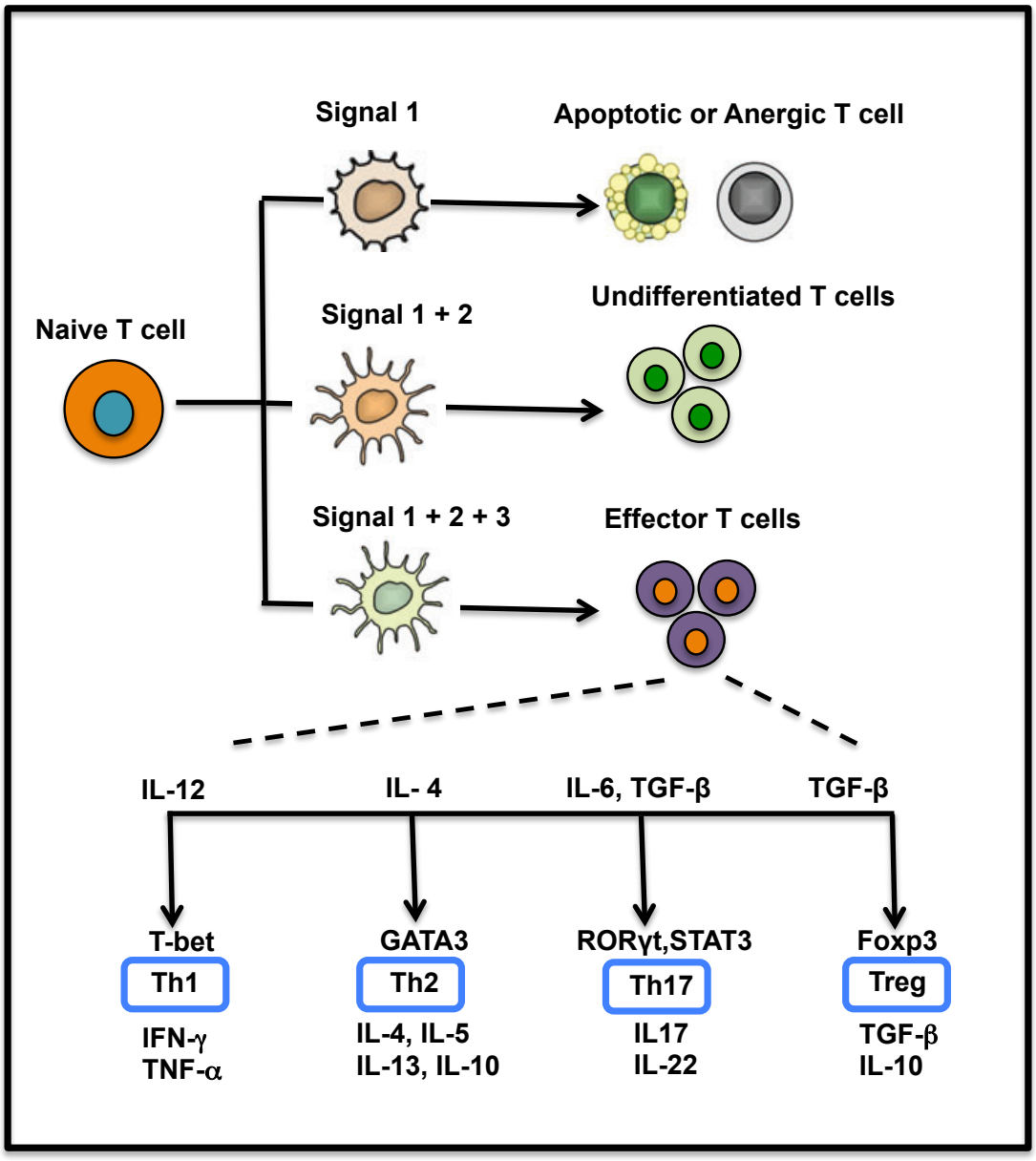

Figure 2. Activation of $CD4^+$ T cells into Th cell lineages. Signal 1 (antigen presentation), Signal 2 (co-stimulation) and Signal 3 (differentiation). Th cell lineages are characterized by expression of lineage-specific transcription factors and the production of distinct effector cytokines. Adapted from (108).

1.7. *Mycobacteria*

Tuberculosis (TB) is a chronic infectious disease caused by *Mycobacterium tuberculosis* (*Mtb*). TB is one of the top ten causes of death in the world and remains therefore a major global health concern (109). Infection is transmitted by the inhalation of aerosols harboring *Mtb*. The live attenuated strain of *M. bovis* called Bacille Calmette-Guérin (BCG) is the only available vaccine against TB, which was approved by the World Health

Organization (WHO) in 1928. Although there is a lot of debate regarding the efficiency of BCG in adults, its role in controlling childhood TB is accepted (110).

1.7.1. BCG, History and Immune response

In 1905 Guerin first found that TB in cattle could be caused by a virulent bovine strain of the tubercle bacillus. In the process of developing BCG as a vaccine for TB, Guérin and Calmette repeatedly cultured the bacillus on different culture media to reduce its pathogenic nature. It was in 1921, after 230 passages, that Guérin and Calmette were confident about the safety of BCG (111). Although the WHO approved BCG as a vaccine against TB in 1928, the Lübeck disaster in 1930 led to its withdrawal from national vaccination campaigns, until the end of the Second World War, when BCG vaccination was again introduced in various countries (112).

Understanding the immune response to BCG is a prerequisite for improving BCG on a rationale basis, be it to develop new BCG variants or to implement new vaccination strategies. In clinical practice, BCG is given intradermally in the skin (113), which requires highly skilled practitioners to correctly and reproducibly deliver it. The degree of protection conferred by BCG vaccination against *Mtb* challenge was tested in various animal models using different routes and doses of BCG (114). BCG triggers a Th1 response, an immune response capable of controlling mycobacterial infection (115). Production of Th1 cytokines IFN-γ and TNF-α, also correlates with protective immune responses against TB in humans and mice (116). Clinical studies confirm that BCG dose can influence the frequency and intensity of T cell responses (117). Although, TB is predominantly a lung disease, BCG-mediated protection in the lungs can be initiated to a similar degree by either intranasal or subcutaneous routes of vaccination (118). This suggests that pulmonary protection can be generated even by vaccination in the skin. Indeed, a group of $CXCR3^{+}CCR6^{+}CD4^{+}$ memory T cells are now considered as major players that champion, the recall response to *Mtb* epitopes during challenge (119). However, the importance of bi- and multifunctional $CD4^{+}$ T cells that produce IFN-γ, TNF-α and IL-2 in immune protection against TB is less defined (120).

In addition, a higher dose of BCG administrated intratracheally gives more protection than a lower dose, but the use of a higher dose could lead to an increase in adverse pathology in lung (118). Interestingly, BCG is also used to treat bladder cancer. The anti-tumor

properties of BCG is observed in both humans (121) and in animal models (122, 123). Heat-killed BCG (HK-BCG) has been reported to trigger similar anti-tumor activity as live BCG in the immunotherapy of bladder cancer (124, 125), while live BCG has been argued to be better than HK-BCG in generating a protective immune response against *Mtb* (126).

1.7.2. Recombinant BCG vaccines

Although BCG is protective against childhood TB, its immune protection gradually wanes over time. New strategies are needed for preventing new TB cases. One strategy is to improve BCG vaccine efficacy by using recombinant BCG (rBCG) strains that provide better protection and safety than the parent BCG vaccine. Towards this aim mycobacterial antigens have been introduced and overexpressed in BCG. The immunogenicity and protection of such BCG constructs have been tested in mice. Immunization with rBCG, co-expressing the fusion protein Ag85B-ESAT6-Rv3620c lead to a significant increase in the production of Th1 cytokines IFN-γ, TNF-α and IL-2 (127). Studies were BCG was made to overexpress Ag85A, Ag85B and ESAT-6 generally reveal improved recall responses to the antigens overexpressed but protection against *Mtb* challenge is either lacking or partial (128-130). Together, it suggests that overexpression of *Mtb* antigens in BCG is not enough to improve BCG vaccine efficacy. This also raises concerns about the choice of antigens used in these constructs or the strategy itself.

On the other hand, an rBCG created with the aim of modulating phagocytic cell function has yielded more promising results. This rBCG is an urease C (*ureC*)-depleted BCG that expresses listeriolysin (*hly*) from *Listeria monocytogenes* (BCG ΔureC::hly Hm) is constructed to improve phagosome acidification in infected APCs that in turn assist BCG antigen release and antigen loading onto MHC (131). Pre-clinical studies have shown that mice vaccinated with BCG ΔureC::hly Hm, but not the parental BCG, were better protected against *Mtb* (132). A phase I clinical trial with this rBCG has improved IFN-γ-producing T-cell responses in BCG-naïve and BCG-immunized individuals compared to parental BCG (131).

2. METHOD DEVELOPMENT

To investigate DC migration to DLN and T-cell priming to BCG, requires appropriate functional assays. Methodologies used in this thesis include but not limited to the assays summarized below. For a more detailed description of these and other methods, the reader is referred to the materials and methods of each respective study.

2.1. Migration assays

2.1.1. CFSE – Migration assay: (Paper I – IV)

This assay was developed to identify and quantify the cells migrating from the footpad to the DLN in response to BCG, figure 3. To track migrating cells we injected BCG in the hind footpad of mice and 24hrs before sacrifice, CFSE was injected in same footpad that previously received BCG. Flow cytometry was used to characterize CFSE-labeled cells in the DLN, the popliteal LN. In certain experiments, confocal microscopy was used to investigate the distribution of CFSE-labeled cells in the DLN. Unlike FITC skin painting that measures cumulative cell migration from skin to DLN in response to topical application of contact sensitizers, the CFSE migration assay enables the identification of cells migrating from skin to DLN within a defined, 24hr period, in response to an injected stimulus, in our case BCG. The assay is discussed in more detail in the discussion section of this thesis and in paper IV.

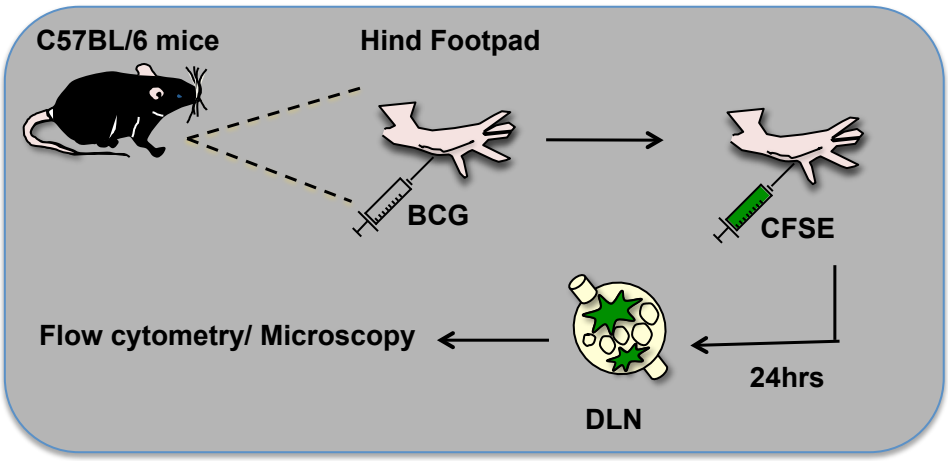

Figure 3. CFSE – Migration assay showing footpad injection with BCG followed by CFSE injection into same footpad, which previously got BCG. Analysis of DLN for immune cells through flow cytometry and microscopy.

2.1.2. Adoptive transfers: (Paper I, II & IV)

Numerous studies on adoptive transfer strategies have been useful in addressing not only migration of DCs but also the activation and expansion of antigen-specific T cells *in vivo*. Adoptive transfer experiments with DCs were performed to study the contribution of DC-extrinsic and -intrinsic factors in the relocation of DCs to the DLN after BCG infection. The above is achieved by labeling BMDCs with CFSE *in vitro* and investigating the frequency of CFSE-labeled BMDCs in DLN by flow cytometry figure 4 (paper I and IV). On the other hand, adoptive transfer experiments with mycobacteria Ag85B-specific P25 TCRTg T cells allowed us to investigate activation markers, proliferation and cytokine production by BCG-antigen specific CD4+ T cells *in vivo*, also by flow cytometry.

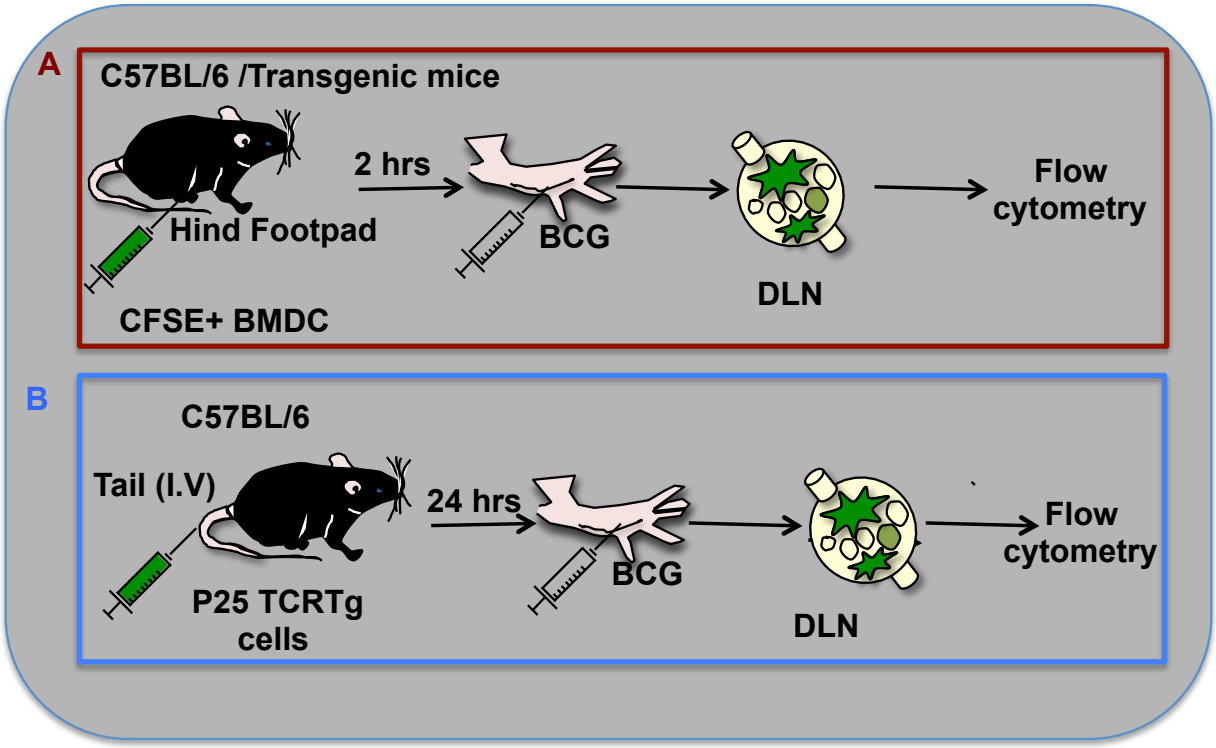

Figure 4. Adoptive transfer includes (A) footpad injection of CFSE-labeled BMDCs into C57BL/6 Followed by BCG injection in the footpad and analyzing the immune cells by flow cytometry. (B) intravenous (I.V) injection of CFSE-labeled P25 TCRTg cells into the tail vein of C57BL/6. Followed by BCG injection in the footpad and analyzing the immune cells by flow cytometry.

2.2. DC- T cell co-culture assay: (Paper II)

In vitro co-culture assays were performed with splenic DCs and naïve P25 TCRTg cells to investigate the impact of *H. polygyrus* on the priming of BCG-specific $CD4^+$ cells. DCs were conditioned with either excretory–secretory product (HES) or soluble worm Ag (SWAg) from H. *polygyrus* for 2hrs and subsequently co-cultured with P25 TCRTg cells at a ratio of 1:5 (DC: T-cell) followed by BCG infection at a multiplicity of infection of 1. IFN-γ was measured in culture supernatants after 5 days. This assay allows one to investigate the effect of soluble molecules from *H. polygyrys* on BCG-triggered production of IFN- γ from T cells, which is otherwise difficult to address *in vivo*.

2.3. Delayed type hypersensitivity (DTH): (Paper II)

A DTH response to mycobacteria can be studied in mice by measuring the swelling of the mouse footpad after injection of purified protein derivative from *Mtb* (PPD). This is similar to the tuberculin skin test in humans used to assess BCG vaccination or TB infection status (133, 134). In our studies, mice were injected with BCG in the hind footpad, and 2 weeks later the other footpad was injected with PPD. The magnitude of DTH was estimated in the PPD-injected footpad by recording the swelling over a period of 72hrs. This assay was adapted to investigate the effect of worm antigens on PPD-triggered DTH response *in vivo*.

3. DISCUSSION OF OBTAINED RESULTS

Initiating the immune response in the LN is a complex process. It requires many steps and cellular interactions. An important step in this process is the transport of microbial components from the site of infection to the DLN. DCs play a major role in the transport of antigen from body surfaces such as skin (1), and are the most potent APC for activating näive T cells. In this part of the thesis our model and several of the main findings obtained in our model are discussed. The reader is referred to each paper for a more detailed description of experiments.

This thesis focuses on DC migration and its consequences for $CD4^+$ T-cell priming to BCG. CFSE, a fluorescent cell-staining dye, was used to label skin cells *in situ* and to track their migration from footpad to DLN over a period of 24hrs (paper I-IV). Clinically, BCG is given intradermally as a vaccine against tuberculosis, but to inject into the dermis requires skill and training (135). Therefore, these injections may not always be given truly intradermal, but a combination of intradermal and subcutaneous. The same likely happens during a footpad injection. In any case, there are several advantages of injecting BCG in the footpad. First, the immune response is concentrated in the pLN, the primary, DLN (paper I). This makes it easier to study the immune response. On the contrary, there are 2 to 3 auricular LNs in mice and these are not always consistently present (136). Second, one can inject larger volumes in the footpad (10-50 µl) compared to the ear (5-10 µl), which makes the footpad a more flexible site.

In our CFSE-based assay, MHC-II^{high} $CD11c^{+/low}$ skin DCs are a major population migrating in response to BCG. Nevertheless the $CFSE^+$ skin DCs in both infected and control animals in our model had increased expression of co-stimulatory molecules, CD80 and CD86 (paper I) suggesting that migrating skin DCs are activated irrespective of the stimulation, consistent with a previous report (137). Interestingly, there is a reduction in BCG-triggered skin DC migration in mice infected with *H. polygyrus* (paper II). This suggests that an intestinal nematode infection in the intestine can alter BCG-triggered migration of DCs from skin to DLN. In contrast to our findings, neutrophils rather than DCs were found to be a major population relocating to DLN in response to BCG infection in the ear dermis (138). This disparity could be due to the different routes of inoculation (138). The mouse ear might be more prone to neutrophil responses compared to other cutaneous sites such as the footpad. Indeed, neutrophils are rapidly recruited to the mouse ear after intradermal *Leishmania major*

infection or the simple inoculation of a needle to the ear (139). Inoculation of *Toxoplasma gondii* in the ear also triggers rapid neutrophil swarming in ear DLNs (140). In the study by Abadie et al, the authors did not find co-localization between $CD207^+$ cells in the skin with BCG (138). They interpreted this as an absent role for migratory DCs in transporting bacilli to the DLN. Since EpCAM (CD326) and langerin (CD207) stain similar DC subsets, and since we found $EpCAM^{low}$ $CD11b^{high}$ skin DCs to be a major cell population relocating to DLN in response to BCG, it is possible that the authors simply missed this population in their study. In line with our findings, a study with Kaede-transgenic mice that express a photo-convertible fluorescence protein also found that $CD11c^+$ DCs moved to DLN in response to BCG (141).

DC migration towards LN is considered an important step in initiating the immune response (1). In line, failure in DC migration leads to poor induction of immunity (142). Lymph-derived DCs are important in maintaining the HEVs and lymphocyte recruitment through lymph in LN (143, 144). BCG arrival to the DLN was a prerequisite for priming mycobacteria-specific P25 TCRTg cells. This is supported by other studies showing that live bacilli in the DLN are needed for the initiation of T-cell responses to mycobacteria (145, 146). It is unclear from our experiments if migratory skin DCs initiate the immune response to BCG by directly presenting antigen to T-cells or indirectly by transferring the antigen to LN-resident DCs. Antigen transfer from migratory skin DCs to LN-resident DCs has been reported for the priming of $CD8^+$ T-cells to Herpes simplex virus (HSV) infection in the skin (147). Studies with *Mtb* suggest that antigen transfer occurs from adoptively transferred, BMDCs to LN-resident DCs in the lung-draining mediastinal LN, and that this optimizes $CD4^+$ T-cell priming to *Mtb* (148).

The number of antigen-bearing DCs that reach the DLN is associated with the magnitude and quality of $CD4^+$ T-cell priming (149), suggesting an important role for migratory DCs in modeling adaptive immune responses. In line with this, blockade of skin DC migration in our model by injection of pertussis toxin (PTx) in the footpad completely ablates proliferation of P25 TCRTg cells and reduces mycobacterial load in the DLN (paper I). Similarly, CCR7-deficient mice, which lack skin-derived DCs and LCs in skin DLN, have impaired T-cell responses to antigens inoculated in the skin (16).

Further, we found that gut infection with the nematode *H. polygyrus* had a negative impact on $CD4^+$ T-cell priming to BCG (paper II). As discussed before, *H. polygyrus* reduced

BCG-triggered skin DCs migration to DLN. These results may explain in part reduced BCG vaccine efficacy and increase susceptibility to mycobacterial infection in individuals carrying worms in their gastrointestinal tract (150). Others have shown that there is an increase in concentration of TGF-β, a regulatory cytokine, in the cerebrospinal fluid and in the serum of *H. polygyrus*-infected mice (151). This increase in TGF-β secretion has been found to be important for establishment of the worm infection by regulating host immune response (152). TGF-β has been implicated in worm-mediated inhibition of several inflammatory diseases (153). It has also been reported that chronic infection with intestinal worms reduce immunity to BCG vaccination in humans and that chronical intestinal worms are associated with increased production of TGF-β by peripheral blood mononuclear cells (PBMC) (154). *H. polygyrus* and its HES are believed to regulate TGF-βR signaling. Interestingly, TGF-β can drive DCs towards a more regulatory phenotype (155-157). However, from *in vitro* cultures we found that HES acts directly on T cells rather than on DCs. Since HES is a complex mixture of molecules (158), more work is needed to investigate its inhibitory effects on cells of the immune system.

Several DC subsets have been reported in mucosa, skin and SLOs (15). By employing previously established markers for identifying subsets of migratory skin DCs (17, 159, 160) we were able to characterize skin DC subsets in our model and identify $EpCAM^{low}$ $CD11b^{high}$ DCs as the main migratory skin DC subset to relocate to DLN in response to BCG (paper I). Indeed, there is an emerging role for migratory $EpCAM^{low}$ $CD11b^{high}$ DCs during infection. This subset has been reported to expand in skin DLN after intradermal infection with BCG or *E. coli* (160) and to promote priming of $CD8^{+}$ T cells to an adenoviral vector delivered via microneedle arrays (161). Another report shows that this subset engulfs *L. major* parasites in the skin (162).

We investigated the molecules that favor the migration of skin DCs to DLN after BCG infection. BCG-triggered skin DC migration is dependent on IL-1R and MyD88 signaling (paper I). MyD88 is an important molecule in mycobacterial-induced DC activation and host resistance to *Mtb* (163). MyD88 is important for both IL-1R and IL-18R signaling but it also signals downstream of several TLRs (27). The phenotype of $MyD88^{-/-}$ and $IL\text{-}1R^{-/-}$ mice infected with *Mtb* clearly suggests that MyD88 signaling downstream of IL-1R signaling is more important than TLR signaling (164). Both DC adoptive transfer and bone-marrow radiation chimera experiments support the requirement for MyD88 in DC migration (paper I). We studied several gene-deficient mice in our CFSE-based migration assay.

Interestingly, we did not find a phenotype for IL-1α$^{-/-}$, IL-1β$^{-/-}$/IL-18$^{-/-}$ or Caspase-1$^{-/-}$ mice (paper IV). There may still be IL-1β release in Caspase-1$^{-/-}$mice, as there is evidence for Caspase-1-independent IL-1β production in *M. tuberculosis*-infected mice (164). Further, comparison of IL-1α$^{-/-}$, IL-1β$^{-/-}$ and IL-1α$^{-/-}$/IL-1β$^{-/-}$ mice during *M. tuberculosis* infection reveals a compensatory role for these cytokines in host resistance (164, 165). We speculate that the observed redundancy of IL-1α and IL-1β in BCG-triggered skin DC migration is indicative of a similar mechanism as that above. Performing the CFSE migration assay with IL-1α$^{-/-}$/IL-1β$^{-/-}$ mice will help clarify this.

The fact that MyD88$^{-/-}$ mice only have a partial phenotype in our model suggests that there must be additional pathways involved in DC influx and BCG entry into DLN. One possible candidate is the cytosolic adaptor molecule Caspase recruitment domain family member 9 (CARD9). CARD9$^{-/-}$ mice fail to mount protective inflammatory responses due to defective production of pro-inflammatory cytokines by myeloid cells (166). This adaptor molecule is important for C-type lectin receptor signaling (167) and regulates production of IL-1β , TNF-α and IL-12p40 during *M. tuberculosis* infection (168).

Inflammation promotes the egress of DCs from tissue. Pro-inflammatory cytokines are produced in response to microbes, contact sensitizers or TLR ligands and are believed to influence the egress of DCs from tissue to DLN (169). Similarly, the production of pro-inflammatory cytokines at the BCG injection site may promote the egress of skin DCs to DLN. In line, we detected mRNA accumulation of IL-1α, IL-1β and TNF-α early after BCG injection in the skin (Paper IV). Studies show that pre-conditioning an injection site in the skin can regulate DC migration to DLN. In particular, administration of TNF-α and IL-1α/β was found to trigger skin DCs migration in other models (81, 170). The above-mentioned approach to improve DC migration was adopted in our model. Injecting the footpad of IL-1R-I$^{-/-}$ mice with IL-12p40 homodimer but not TNF-α restored BCG-triggered skin DC migration. Pre-conditioning the footpad with TGF-β or HES significantly reduced BCG-triggered skin DCs migration to DLN. The latter suggests that TGF-β and HES can impair DC function (155).

BCG dose has also been suggested to be important against *M. tuberculosis* challenge when different routes of BCG vaccinated were compared (114). When we investigated the importance of dose in skin DC migration to DLN, we found that using 10 times less BCG

than our standard dose lead to a reduction in skin DC migration (paper IV). In line, clinical studies suggest that the frequency and intensity of T-cell responses to BCG may be dose-dependent (117).

While historical studies have suggested that the attenuated H37Ra strain of *Mtb* elicits better survival in mice compared with heat-killed H37Ra after challenge with virulent of *Mtb* (126), the mechanisms are not fully understood. The above does not seem to be due to improved DC migration to DLN, since HK-BCG triggers similar relocation of skin DC to DLN as live BCG (paper IV). The originally believed, enhanced effects of live mycobacterial preparations over inactivated mycobacteria may have been overestimated. Indeed, whole-cell lysate preparations of *Mycobacterium* species are currently in the pipeline of TB vaccine development, examples being DAR-901, *M. vaccae* and RUTI (171-173).

HK-BCG has been reported to have similar anti-tumor activity as live BCG in the immunotherapy of bladder cancer (124, 125). Interestingly, inoculation of HK-BCG in the footpad skin induces a stronger expansion of P25 TCRTg cells in the DLN compared to live BCG. This is at least true during the peak of the response to live BCG. Time-course experiments need to be performed to provide additional information on the difference between these inoculations. It has been recently shown that EsxH from the *Mtb* type VII secretion system inhibits antigen processing in macrophages and DCs (174). The inability of HK-BCG to secrete inhibitory effector molecules could explain why it is better than live BCG in triggering T-cell expansion *in vivo*. It is also possible that soluble mycobacterial products (more abundant in a HK-BCG preparation) gain direct access to lymphatics after being injected in the footpad and as such become readily available to LN-resident DCs in the DLN. This remains to be investigated.

In summary, this thesis reports the discovery of a migratory skin DC sub-population that relocates to DLN in response to BCG. This population relocates together with bacilli in an IL-1R-MyD88-dependent manner. A redundancy exists between IL-1α and IL-1β is this process. Further, a chronic intestinal nematode infection impairs BCG-triggered responses including skin DC migration to DLN. Skin DC migration is BCG-dose dependent and does not require viable bacilli. These findings on the early sequence of events that lead to T-cell priming in the DLN may be relevant in vaccine development against TB and in DC-based immunotherapy.

4. CONCLUSION & FUTURE PERSPECTIVES

We found EpCAM^{low} CD11b^{high} cells to be the main skin DC sub-population that migrates to DLN in response to BCG injection in the skin (paper I). This is also in part supported by data from paper II. In line with our findings, other reports suggest that a similar population migrates from skin to DLN after *E. coli*, BCG (160), chemical stress or mechanical injury (20). Our observations also indicate the importance of these migrating DCs in transporting live BCG to the DLN to trigger CD4^{+} T-cell priming (paper I). This however does not establish EpCAM^{low} CD11b^{high} DCs as DCs that champion the priming CD4^{+} T cells to BCG or other mycobacteria for that matter. This could be addressed in different ways.

The antigen-presentation capacity of this as well as other DC subsets from skin DLN could be investigated with naïve P25 TCRTg cells in antigen-presentation assays, similar to those previously performed with gB-I TCRTg cells and HSV-1-infected LN DCs (175). Another approach would be to perform adoptive transfer experiments of BCG-pulsed DCs into MHC-II$^{-/-}$ mice previously injected with naïve P25 TCRTg cells. The transfer into MHC-II$^{-/-}$ recipients would control for antigen-leakage. This approach would allow one to study the ability of different DC subsets in priming T cells *in vivo*, but is potentially limited by the DC numbers needed to perform these experiments. A third way forward could be to study the priming of P25 TCRTg cells in mice where DC subsets are absent or can be depleted *in vivo*, as recently studied during infection with *Candida albicans* (176). Recent advances in single-cell whole transcriptome analysis (177-179) would however allow profiling of both sparse and heterogeneous skin DC subsets. Such analyses could be attempted both from DCs isolated from BCG-stimulated LNs *in vivo*, or from *in vitro*-activated subsets.

Another highlight of this thesis is that skin DC migration and BCG entry into DLN is regulated by IL-1R-MyD88 signaling (paper I). Interestingly, the ligands for IL-1R, IL-1α and IL-1β, were found to be redundant in this relocation of cells and bacilli to DLN (paper IV). Also, the requirement for IL-1R and MyD88 seem to be partial. This is interesting since IL-1R and MyD88 are known to be important regulators of DCs and inflammation. Additional pathways or other, yet unknown, compensatory mechanisms may be happening in our model. This is not easy to investigate. One possibility is to screen gene-targeted mice in the CFSE-based migration assay. Also interestingly, injection of the footpad with PTx prior to BCG injection reveals a requirement for migratory skin DCs in T-cell priming. This treatment totally blocked DC migration and inhibited expansion of naïve P25 TCRTg cells in

the DLN. However, PTx treatment did not ablate mycobacterial entry into DLN, suggesting that some mycobacteria seem to access lymphatic vessels and reach the DLN in the absence of DC (or other cellular) transport, One way to investigate the contribution of such lymph-borne, "cell-free" relocation of BCG to DLN is by injecting BCG directly into lymphatic vessels and studying the outcome of this inoculation route on the priming of P25 TCRTg cells.

Another interesting observation was that HK-BCG seems to be superior to live BCG at priming P25 TCRTg cells in the DLN. This is somewhat contradictory to the belief that live BCG is better than killed BCG at generating protective immune responses to *Mtb* (126). However, several lysate/heat-inactivated-based mycobacterial preparations are currently being tested against TB (109) The outcome of immunization with live versus killed mycobacteria merits further investigation. We speculate that after injection of HK-BCG, P25 TCRTg cells are primed mainly by LN-resident DCs that gain quicker access to free antigen through lymphatics, possibly involving also the LN conduit system (21). This could be studied as previously mentioned in the Results and Discussion section, in experiments using PTx or $CCR7^{-/-}$ mice.

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